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Novel Assay for the Detection of Immunoglobulin G Antihuman Immunodeficiency Virus in Untreated Saliva and Urine

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Epidemiological evidence and laboratory studies indicate that human immunodeficiency virus type 1 (HIV 1) is rarely, if ever, transmitted in saliva or urine. In that both specimens are easy to collect, each may be a useful alternative to serum specimens for anti-HIV screening. A rapid, simple, and robust IgG-capture enzyme-linked immunsorbent assay (GACELISA) suitable for the detection of anti-HIV 1 and 2 in saliva and urine was developed. Following optimisation of the assay, 177 salivary and 568 urine specimens collected from individuals of known serostatus were investigated. The assay was 100% sensitive on 50 salivary (median OD/CO = 8.9) and 126 urinary (median OD/CO = 8.6) specimens collected from anti-HIV-positive patients. The specificity was 100% on 127 salivary specimens (median OD/CO = 0.37) and 422 urinary specimens (median OD/CO = 0.39) collected from anti-HIV-negative individuals. These findings demonstrate that GACELISA HIV 1 + 2 tests on saliva or on urine are an accurate alternative to a conventional anti-HIV test of blood. This assay is satisfactory for surveillance purposes and, with appropriate precautions, could be used clinically.

KEY WORDS: IgG capture ELISA, HIV1 + 2 antibody testing, HIV surveillance

INTRODUCTION

Saliva is a mixture of secretion from the salivary glands and transudate from the capillary bed beneath the buccal mucosa, especially that referred to as gingival crevicular fluid, which flows from the crevice between the gum margin and the tooth. Crevicular fluid has an antibody content similar to that of plasma, with IgG, IgM, and IgA immunoglobulin classes present [Brandtzaeg et al., 1970; Challacombe et al., 1978], but, once mixed with salivary gland secretion, it becomes greatly diluted. The IgG concentration of whole saliva is thus ~1,000-fold less than that of plasma [Roitt and Lehner, 1983].

The immunoglobulin concentration of urine has been less fully documented than that of saliva. It is determined by plasma protein excretion through the kidneys and possibly by transudation through the walls of the urinary tract. The physiological concentration of IgG in urine, i.e., when renal function is normal, is ~10,000-fold less than that in plasma [Connell et al., 1990].

Epidemiological evidence and laboratory studies suggest that human immunodeficiency virus type 1 (HIV 1) is not transmissible in saliva [Fischl et al., 1987; Archibald and Cole, 1990] or urine [Skolnik et al., 1989; Cao et al., 1990], and, in that both specimens are easy to collect, each may be a useful alternative to a serum specimen for surveillance and for diagnostic investigations. The presence of anti-HIV in saliva was first described by Archibald and colleagues [1986], who found IgA anti-HIV in 42 of 48 HIV-infected subjects. However, Parry and coworkers [1987] demonstrated, using simple solid-phase assays, that IgG anti-HIV was also present in the saliva of all 43 HIV-infected subjects examined and at higher levels than IgA anti-HIV.

In 1988, Johnson and colleagues compared assays for anti-HIV detection in saliva and serum specimens. Of those applied, only an IgG-capture radioimmunoassay (GACRIA) was 100% sensitive. Some of the commercial anti-HIV assays investigated had specificities approaching that of GACRIA, but they were less sensitive. Other workers [Major et al., 1991; Behets et al., 1991; Shoeman et al., 1989] have found that commercial EIAs and Western blots can be modified to give specificity comparable to GACRIA when saliva is tested, but still they are less sensitive. So far the detection of anti-HIV in urine by conventional EIA is relatively insensitive and has often involved preliminary

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concentration and clarification of the specimen [Cao et al., 1988, 1989; Reagan et al., 1990; Desai et al., 1991].

We now describe the development of an IgG antibody-capture ELISA (GACELISA) for the detection of anti-HIV 1 and 2 in saliva and urine, which offers an alternative to serum assays and has overall advantages of safety, convenience, and cost reduction. It has proved especially useful for epidemiological studies in high-prevalence areas and for investigating groups who may not tolerate venepuncture, such as intravenous drug users and children.

SPECIMENS AND METHODS

Specimens

Optimisation panel. A panel of six specimens was prepared to ascertain the optimal conditions for the assay. This consisted of two sera, one unreactive and the other weakly reactive for anti-HIV; two saliva samples, one from an anti-HIV-negative and one from an anti-HIV-positive individual (the level of reactivity of the latter specimen was determined by GACRIA [Parry et al., 1987], a proven method for anti-HIV detection in saliva); and two urine samples, one from an anti-HIV-negative individual, the other an anti-HIV weakly reactive urine sample manufactured by diluting an anti-HIV-positive sample in pooled anti-HIV-negative urine.

Collection of clinical specimens. Salivary specimens were collected simultaneously with serum from 127 anti-HIV-negative and 50 anti-HIV-positive individuals. Salivary specimens were collected by Salivette (Sarstedt, Leicester, U.K.). A Salivette consists of a cylindrical roll of compressed absorbent material within a plastic container, which has a small hole in the bottom. This container is enclosed in a plastic centrifuge tube. The subject is asked to chew gently on the absorbent roll so that saliva is taken up in it. After replacing the absorbent roll in the internal container, the whole device is returned to the laboratory in the centrifuge tube. There the tube is centrifuged to release the saliva from the roll to be recovered from the bottom of the tube. These salivary specimens were stored at 4°C until tested and thereafter at −20°C or below.

Urine specimens were collected from 442 individuals known to be anti-HIV negative and 126 known to be anti-HIV positive. They were collected in sterile containers, and the majority were stored at 4°C, although samples collected in Africa were frozen there and during shipment.

Optimisation of GACELISA Conditions

GACELISA utilises modular microplates (12 strips of eight wells) in which wells are precoated with antihuman IgG (gamma-chain specific). Sample, conjugate, and a signal development system (IQ Bio amplification system; Novo Nordisk Diagnostics Ltd, Cambridge, U.K. [Self, 1985]) employed at optimal conditions (Murex Diagnostics, Dartford, U.K.) (unpublished) are each added with intervening washing steps (Fig. 1). Finally, 2 M H_2SO_4 is added to stop the colour reaction.

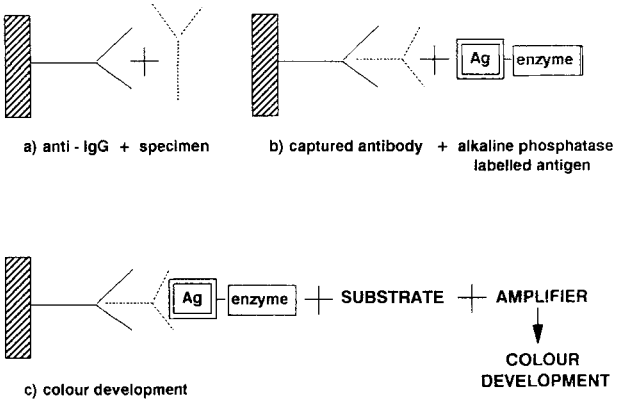

Fig. 1. GACELISA. Addition of specimen (30 min) **(a)**, addition of HIV 1 + 2 conjugate (60 min) **(b)**, and colour development (30 min) **(c)**.

Several procedural variables were investigated using the optimisation panel.

Preparation of the Solid Phase

The optimum concentration of antihuman IgG (Dako Ltd., High Wycombe, U.K.) for coating the solid phase was determined by titration against known anti-HIV-positive and -negative samples. The anti-IgG was diluted in a carbonate/bicarbonate solution, pH 9.6 (1.6 g $NaCO_3$, 2.94 g $NaHCO_3$ in 1,000 ml of freshly distilled water) and added in 100 µl volumes to U bottomed microtitre wells (U8 maxisorp, 4-75078A; Life Technologies, Paisley, Scotland). Adsorption of antihuman IgG to the solid phase after incubation for 1 hr at 37°C followed by overnight incubation at 4°C was compared with that after incubation overnight at room temperature.

A number of blocking agents were investigated, including dried milk (Marvel, Premier Brands UK Ltd., Adbaston, U.K.), 0.05% Tween 20 in phosphate-buffered saline with and without the addition of fetal bovine serum or bovine serum albumin, and 5% Sol-u-pro (Dynagel Inc., Illinois) in distilled water. The minimum time needed to block the surface of the plates, the effect of drying them by inversion for 24–72 hr in a hot room at 37°C, and their stability when kept in a plastic bag with a desiccant sachet were investigated.

Sample Incubation

Sample incubations of 10, 20, 30, and 60 min at 37°C were investigated.

Conjugate Incubation

We initially investigated an expression product of recombinant DNA in which the gp41 and p24 genes of HIV 1 were joined and, following purification of the expression product, conjugated to alkaline phosphatase (Murex Diagnostics, Dartford, U.K. [Wellcome Foundation]).

Subsequently, a combined HIV 1 + 2 conjugate became available that incorporated the HIV 1 recombi-

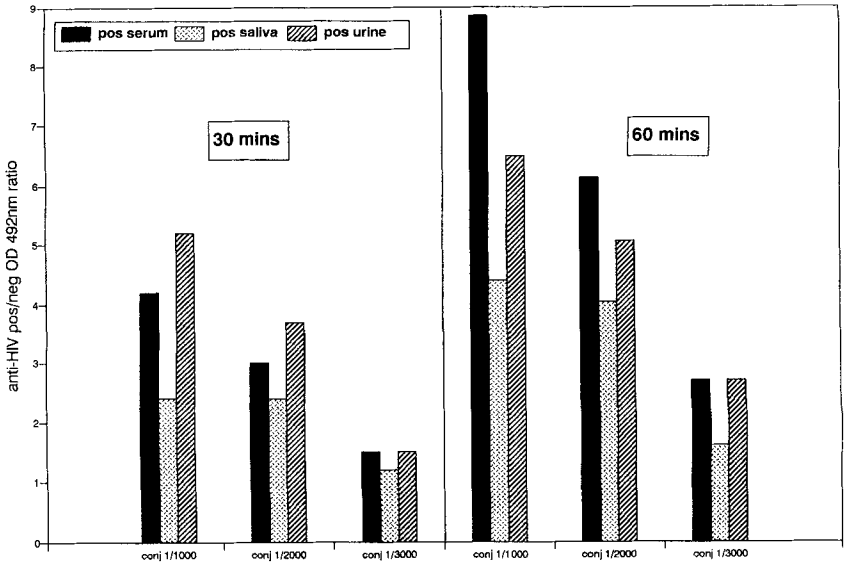

Fig. 2. Effect of conjugate dilution and incubation time at 37°C on discrimination between anti-HIV-positive and anti-HIV-negative specimens of serum, saliva, and urine.

nant DNA (gp41, p24) expression protein and a synthetic oligopeptide representing the immundominant region of the gp36 transmembrane protein of HIV 2. The ability of the two conjugates to discriminate between positive and negative samples in the optimisation panel was compared as a preliminary to the use of HIV 1 + 2 conjugate to investigate clinical samples.

Controls

Because of the possible instability of diluted saliva and urine specimens used as weak positive controls, and because salivary controls are difficult to obtain in adequate volume, serum controls were substituted for them. Separate anti-HIV 1- and anti-HIV 2-positive controls were prepared from confirmed anti-HIV-positive serum samples. These were filtered and diluted (1/50 for anti-HIV 1 and 1/8 for anti-HIV 2) in filtered serum known to be anti-HIV negative. When tested in the GACELISA, these dilutions gave optical density (OD) values at a wavelength of 492 nm of ~2.0. They were then further diluted 1/800 for anti-HIV 1 and 1/400 for anti-HIV 2 in a diluent that did not contain human serum. The bulk volumes were retested to ensure that OD signals of ~2.0 were maintained. These controls were compared for long-term stability at −20°C, 4°C, room temperature and 37°C in the dark and at room temperature in the light. The anti-HIV-negative control was a defibrinated and delipidised serum prepared by Murex Diagnostics.

RESULTS

Optimisation of Assay Conditions

Preparation of the solid phase. Adsorption of anti-IgG to the solid phase was equally effective whether samples were incubated for 1 hr at 37°C followed by overnight incubation at 4°C or incubated overnight at room temperature.

Five percent Sol-u-pro was the most effective blocking agent, reducing the nonspecific binding without affecting the specific signal. Incubation for 60 min at room temperature was necessary to block sufficiently; longer incubation had no additional effect.

Plates stored dry at 4°C in a plastic bag in the presence of desiccant were compared with freshly prepared, wet plates blocked either with 5% Sol-u-pro for 1 hr or with 5% dried milk in phosphate-buffered saline (containing 1% bovine serum albumin, which was initially shown to be the best blocking agent). The plates stored dry were found to give the best discrimination between anti-HIV-negative and -positive samples. These plates were stable for at least 6 months at 4°C.

Sample incubation. Sample incubation for 10 min at 37°C was sufficient to attain maximum reactivity even with weakly reactive samples in the optimisation panel. However, it was decided that a 30 min period would be more convenient.

Conjugate incubation. Conjugate incubation for 60 min was optimal, with inferior results obtained at 30 min (Fig. 2). The results obtained when the optimisation panel was tested in parallel with the HIV 1 and the HIV 1 + 2 alkaline phosphatase conjugates were similar.

Clinical Evaluation

Cut-off determination. The mean OD value for the anti-HIV-negative control over the period of the investigation was 0.116 (102 observations). This corresponds closely to the mean OD values obtained when testing the 127 saliva (0.12) and 442 urine (0.13) specimens from anti-HIV-negative subjects.

TABLE I. Mean, Median, and Range of GACELISA Reactivities (OD:CO) of Seropositive and Seronegative Individuals

	Anti-HIV serostatus	No.	Mean	Median	Range
Salivette	+	50	8.6[a]	8.9	1.5–11.2
	–	127	0.41	0.37	0.27–0.90
Urine	+	126	8.0	8.6	1.1–13.2
	–	442	0.40	0.39	0.17–0.99

[a]Ratio of test optical density: cut-off (OD:CO). Cut-off is mean neg control + 0.2.

After analysis of the results, it was decided that a value of 0.2 (equivalent to 5 standard deviations of the mean of the negative samples) should be added to the mean OD of the negative controls to calculate the cut-off. This cut-off gave excellent discrimination between saliva and urine specimens collected from anti-HIV-positive subjects and those collected from anti-HIV-negative subjects.

Investigation of IgG anti-HIV in saliva. When 177 salivary specimens (collected by Salivette) were examined by GACELISA, there was 100% agreement with serological tests on the same individuals (Table I). Clear discrimination was found between the reactions obtained when Salivette specimens collected from subjects with serologically confirmed HIV infection and those collected from uninfected subjects were tested (Fig. 3): Only three of 50 Salivette specimens from infected subjects gave an OD/CO below 6.0. Furthermore, a comparison of salivary reactivity with serum reactivity showed, in most cases, a close similarity of the GACELISA OD/CO for the Salivette to that of the matched serum sample (see Fig. 5).

Investigation of IgG anti-HIV in urine. There was 100% agreement between serological and urinary results on 568 individuals investigated (Table I). Urine specimens from 113 (89%) of the 126 seropositive subjects gave OD values over three times the cut-off value (Fig. 4). A further comparison was made of the OD/CO values obtained when testing simultaneously collected serum and urine samples from 28 anti-HIV-positive subjects (Fig. 5). This showed that the serum usually gave a stronger reaction than the urine specimen. Some of the urine specimens, however, had been frozen prior to testing.

DISCUSSION

We have developed a rapid, robust enzyme immunoassay that detects IgG anti-HIV in untreated salivary and urinary specimens with a sensitivity and specificity comparable to that of conventional assays on serum. GACELISA HIV 1 + 2 thus provides a satisfactory alternative to collecting and testing blood specimens. It should be particularly useful for epidemiological surveillance and investigation of patients unwilling to give blood samples. Furthermore, GACELISA displays in tests on serum specimens equivalent sensitivity to other currently available anti-HIV enzyme immunoassays (data to be published).

Salient features of the optimisation of the assay were that the solid phase was best blocked with 5% Sol-u-pro; that the sample incubation could be as short as 10 min even though in practice a longer incubation time might be more convenient; that the HIV 1 and HIV 1 + 2 conjugates were equivalent; and that for best results conjugate incubation should be for 30, and preferably 60, min at 37°C. Employing a cut-off value of the mean negative control OD +0.2, it was shown that 100% sensitivity and specificity were achieved when testing 177 salivary samples collected by Salivette. With four exceptions, there was close correlation between the strength of reaction obtained when testing the Salivette specimen and the simultaneously collected serum. Although the number of Salivette specimens evaluated by us was relatively small, GACELISA has also been used successfully by others to investigate Salivette specimens and constituents of saliva (E.A.C. Follett, personal communication). Follett observed no false reactions in 122 Salivette samples from uninfected subjects and detected anti-HIV in specimens of whole saliva (123/123), parotid saliva (121/121), crevicular fluid (124/127), and submandibular saliva (97/99) of HIV-seropositive individuals.

Of the urine samples collected from 126 anti-HIV-positive subjects, 113 (89%) gave OD values greater than three times the cut-off. The reactions of nine samples (7%) were, however, clustered between the cut-off and twice the cut-off. These weakly reactive urine samples were frozen before and/or during transport from Africa to the laboratory, and this probably contributed to the low level of anti-HIV reactivity. It is essential that urine samples are not frozen when antibody testing is intended. We have demonstrated a decrease in the specific anti-HIV signal of urine samples following experimental freezing and thawing, and we thus recommend their storage at 4°C.

Because of the rarity of HIV 2 infection in the United Kingdom [Anonymous, 1992] it was not possible in this study to evaluate the sensitivity of HIV 1 + 2 GACELISA on clinical samples. However, in another study, saliva and urine specimens collected from HIV 2-infected subjects were all reactive in GACELISA [Gershy-Damet et al., 1992].

The sensitivity of GACELISA on specimens such as saliva and urine with low or very low immunoglobulin concentrations may present a problem in confirming the presence of anti-HIV in such specimens that are reactive. GACRIA is an adequate confirmatory test for saliva and Salivette specimens [Parry et al., 1987; Johnson et al., 1988], but it cannot be made widely available and is not as sensitive as GACELISA. We have used an IgG-capture modification of the Serodia-HIV particle-agglutination assay (GACPAT) to confirm reactions on urine specimens [Parry and Mortimer, 1989; Connell et al., 1990], but it is not suitable for saliva specimens. Neither of these confirmatory approaches is ideal, because in both cases the same "cap-

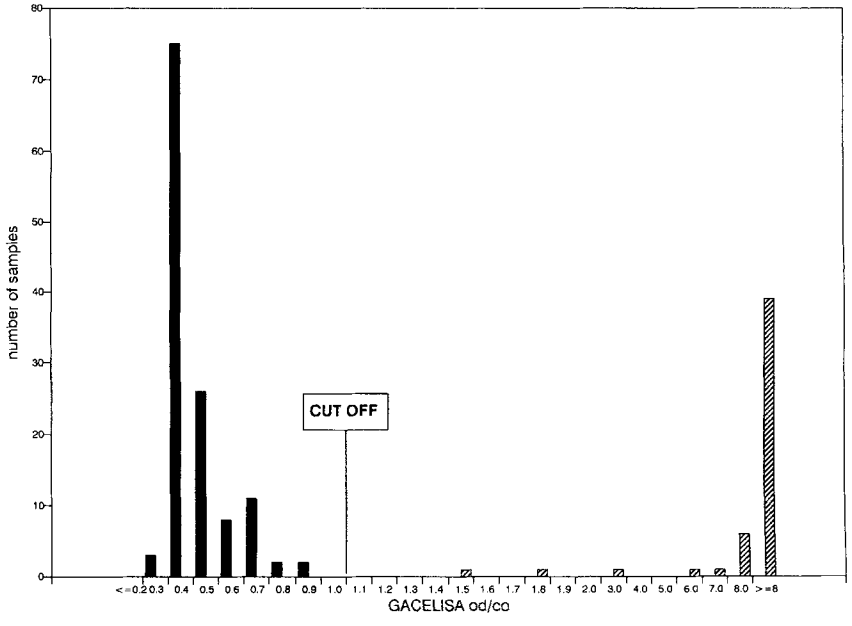

Fig. 3. Distribution of GACELISA reactions (optical density/cut-off ratio) for Salivette specimens collected from 50 seropositive (hatched bars) and 127 seronegative (solid bars) individuals.

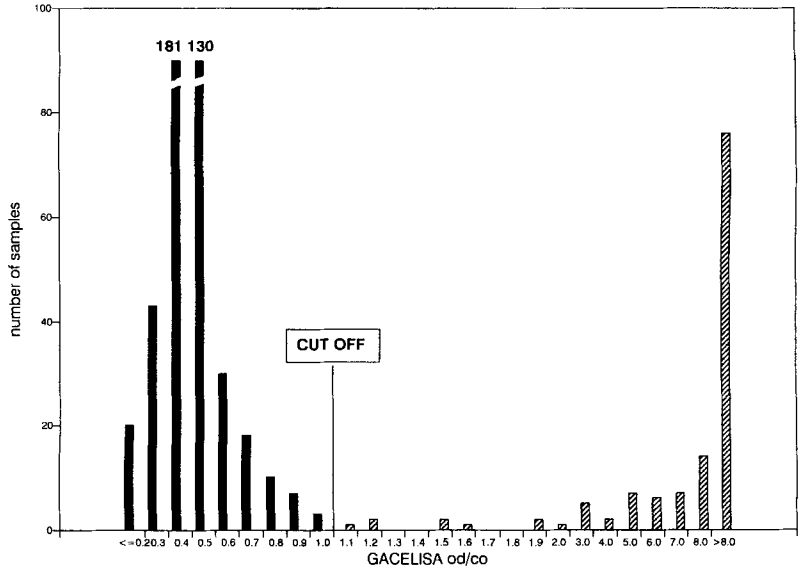

Fig. 4. Distribution of GACELISA reactions (optical density/cut-off ratio) for urine specimens collected from 126 seropositive (hatched bars) and 442 seronegative (solid bars) individuals.

ture" format is being used for screening and for confirmatory testing.

For samples that give weak reactions in GACELISA but equivocal reactions in GACRIA or GACPAT, a modified Western blot (a 1:5 dilution of saliva or 1:2 dilution of urine as sample and the incubation period increased to 48 hr) may facilitate confirmatory testing. However, the number of HIV-specific bands observed when urine from an HIV-infected individual is tested is often less than when the same individual's serum specimen is tested. Frequently only gp120/160 bands are seen [Connell et al., 1990; Shoeman et al., 1989]. As an alternative to confirmatory tests on GACELISA reactive saliva and urine specimens, individuals can be asked to provide a blood specimen for confirmatory testing.

Investigation of anti-HIV in saliva and urine by GACELISA HIV 1 + 2 has convenience, cost, and

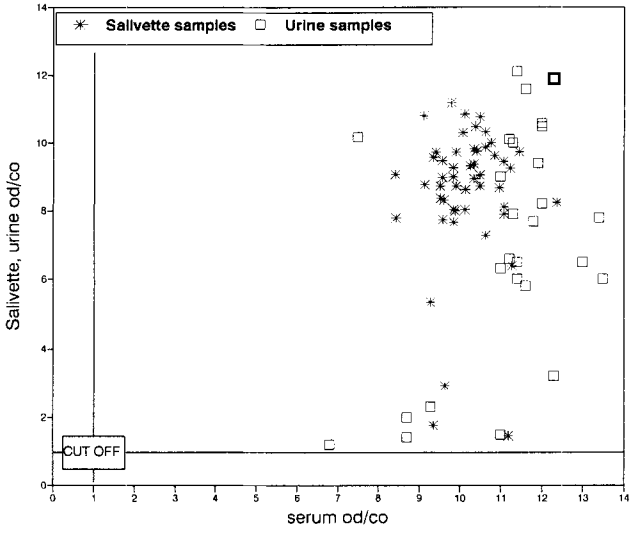

Fig. 5. Comparison between the optical density/cut-off ratios obtained with anti-HIV-positive serum specimens and those obtained with a Salivette or urine specimen collected from the same individual at the same time and tested in parallel by GACELISA HIV 1 + 2.

safety benefits and provides the opportunity to study HIV prevalence in groups previously inaccessible because blood samples could not be obtained. It may soon be possible to use GACELISA for the clinical assessment of HIV status, although further evaluation, especially of the reliability of saliva collection methods, specimen storage, and seroconversion sensitivity, will be necessary.

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REFERENCES

Anonymous (1992): AIDS and HIV 1 infection in the United Kingdom: monthly report. Communicable Disease Report 2:17–20.

Archibald DW, Cole GA (1990): In vitro inhibition of HIV-1 infectivity by human saliva. AIDS Research and Human Retroviruses 6:1425–1432.

Archibald DW, Zon L, Groopman JE, McLane MF, Essex M (1986): Antibodies to human T-lymphotropic virus type III (HTLV-III) in saliva of acquired immunodeficiency syndrome (AIDS) patients and in persons at risk for AIDS. Blood 67:831–834.

Behets FM, Bazapeyo E, Quirn TC, Atikala L, Bishagara K, Nzila N, Lago M, Piot P, Ryder RW, Brown CC (1991): Detection of salivary HIV-1 specific IgG antibodies in high-risk populations in Zaire. Journal of Acquired Immunodeficiency Syndrome 4:183–187.

Brandtzaeg P, Fjellanger I, Gjeruldsen ST (1970): Human secretory immunoglobulin in salivary secretions from individuals with normal or low levels of serum immunoglobulin. Scandinavian Journal of Haematology 12(Suppl 1):1–83.

Cao Y, Friedman-Kien AE, Chuba JV, Mirabile M, Mosai B (1988): IgG antibodies to HIV-1 in urine of HIV-1 seropositive individuals. Lancet i:831–832.

Cao Y, Friedman-Kien AE, Mirabile M, Li XL, Alam M, Dieterich D, Ho DD (1990): HIV-1 neutralising antibodies in urine from seropositive individuals. Journal of Acquired Immunodeficiency Syndrome 3:195–199.

Cao Y, Hosein B, Borkowsky W, Mirabile M, Baker L, Baldwin D, Poiesz BJ, Friedman-Kien AE (1989): Antibodies to Human Immunodeficiency Virus Type 1 in the urine specimens of HIV-1 seropositive individuals. AIDS Research and Human Retroviruses 5:311–319.

Challacombe SJ, Russell MW, Hawkes JE, Bergmerer LA, Lehner T (1978): Passage of immunoglobulin from plasma to the oral cavity in rhesus monkeys. Immunology 35:423–31.

Connell JA, Parry JV, Mortimer PP, Duncan RJS, McClean KA, Johnson AM, Hambling MH, Barbara J, Farrington CP (1990): Accurate assays for anti-HIV in urine. Lancet 335:1366–1369.

Desai S, Bates M, Michalski FJ (1991): Detection of antibody to HIV-1 in urine. Lancet 337:183–184.

Fischl MA, Dickinson GM, Scott GW, Klimas N, Fletcher MA, Parks W (1987): Evaluation of heterosexual partners, children and household contacts of adults with AIDS. JAMA 257:640–644.

Gershy-Damet GM, Koffi K, Abouya L, Sasson-Moroko M, Brattegard K, Parry JV, de Cock KM, Mortimer PP (1992): Salivary and urinary diagnosis of HIV 1 and 2 infection in Cote d'Ivoire by two assays. Transactions of the Royal Society of Tropical Medicine and Hygiene 86:670—671.

Johnson AM, Parry JV, Best SJ, Smith AM, de Silva M, Mortimer PP (1988): HIV surveillance by testing saliva. AIDS 2:369–371.

Major CJ, Read SE, Coates RA, Francis A, McLaughlin BJ, Millson M, Shepherd F, Farring M, Calzavara L, MacFadden D, Johnson JK, O'Shaughnessy MV (1991): Comparison of saliva and blood for human immunodeficiency virus prevalence testing. Journal of Infectious Diseases 163:699–702.

Parry JV, Mortimer PP (1989): An immunoglobulin G antibody capture particle adherence test (GACPAT) for antibody to HIV-1 and HTLV I that allows economic large scale screening. AIDS 3:173–176.

Parry JV, Perry KR, Mortimer PP (1987): Sensitive assays for viral antibodies in saliva: An alternative to tests on serum. Lancet ii:72–75.

Reagan KJ, Lile CC, Book GW, Devash Y, Winslow DL, Bincsik A (1990): Use of urine for HIV-1 antibody screening. Lancet 335:358–359.

Roitt I, Lehner T (1983): Oral immunity. In: Immunology of Oral Diseases, 2nd ed. Oxford: Blackwell Scientific Publications, pp 279–304.

Self CM (1985): Enzyme amplification—A general method applied to provide an immuno assisted assay for placental alkaline phosphatase. Journal of Immunological Methods 76:389–393.

Shoeman RL, Pottathhill R, Metroka C (1989): Antibodies to HIV in saliva. Lancet ii:1145–1146.

Skolnik PR, Kosloff SR, Bechtel LJ, Huskins KR, Flynn T, Kovthas N, McIntosh K, Mirsch MS (1989): Absence of infectious HIV-1 in the urine of seropostive viraemic subjects. Journal of Infectious Diseases 160:1056–1060.

Wellcome Foundation Ltd. Immunoassay for antibody, esp HIV antibody, using recombinant antigens from different organisms in sandwich assay to eliminate false positives. European Patent EP-307-149A.